derivative which gives the diazo reaction was isolated. It contains the acetyl group presumably linked to the oxygen of the hydroxylamino group. The other derivative is acetylated on the nitrogen and cannot be diazotized. The application of this reaction to the detection of hydroxylaminobenzenesulfonamide in body fluids is discussed. The preparation of 4-nitrosobenzenesulfonamide is described.

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[CONTRIBUTION FROM THE BAKER LABORATORY OF CHEMISTRY AT CORNELL UNIVERSITY]

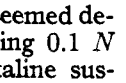
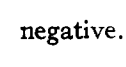
Gliotoxin, the Antibiotic Principle of *Gliocladium fimbriatum*. II. General Chemical Behavior and Crystalline Derivatives[1]

BY WILLIAM F. BRUCE, JAMES D. DUTCHER,[2] JOHN R. JOHNSON AND LEON L. MILLER[3]

In a preliminary examination of the chemical behavior of gliotoxin, Weindling[4] has observed that in spite of its nitrogen content, gliotoxin has no basic properties. By prolonged heating with acid, it was slowly converted to a gum from which none of the original material could be recovered. In a partition between ether and 1% potassium hydroxide, practically none of the gliotoxin was dissolved in the alkaline layer. This showed the non-acidic nature of the material. It was, however, rapidly altered by treatment with alkali. Boiling with 5% potassium hydroxide rapidly eliminated sulfur and, even in the ether partition, a large proportion of the material was altered. Gliotoxin was found strongly reducing toward permanganate.

To extend these observations, a quantitative study of the effect of alkali on gliotoxin seemed desirable. By connecting a trap containing 0.1 *N* hydrochloric acid with a refluxing alkaline suspension of gliotoxin, evolution of a volatile base was readily demonstrated. On evaporation of the acid solution, a hydrochloride and from it a chloroplatinate were prepared which showed that the volatile base was methylamine, and in an amount which accounted for about half the nitrogen in the sample. The amount of sulfur liberated as sulfide by the action of alkali was then determined by acidification and steam distillation of the solution which remained. From 40–60% of the total sulfur was liberated as hydrogen sulfide, and some as elementary sulfur. In the non-volatile portion, a red amorphous solid remained, insoluble in acid, but soluble in alkali, containing nitrogen and sulfur. By using barium hydroxide in place of sodium hydroxide, the same volatile products were observed, but the residue was not as highly colored. On purification by sublimation *in vacuo*, it yielded a small amount of crystalline solid. This solid, slightly soluble in water, dissolved readily in sodium bicarbonate solution, and formed a silver salt, methyl and ethyl esters through which it was identified as indole-2-carboxylic acid.

Gliotoxin proved inert toward phenyl isocyanate, either when refluxed in benzene solution or on long standing at ordinary temperature: the original material was recovered unchanged. With diazomethane, methyl iodide and dimethyl sulfate, some reaction may have occurred since none of the original material was recovered; only gums and sirups were obtained.

A Zeisel determination showed the absence of methoxyl or ethoxyl groups. The N-methyl determination[5] was positive in agreement with the isolation of methylamine. No reaction occurred with the carbonyl reagents hydroxylamine, semicarbazide or 2,4-dinitrophenylhydrazine. The Ehrlich reaction, pine splint test, Keller's indole reaction and the ninhydrin reaction were all negative. Clowes' test for  or  using phloroglucinol and 50% sulfuric acid,[6] was also negative. Ammoniacal silver nitrate (Tollens reagent) and phosphotungstic acid (Folin reagent) in the presence of sulfite were reduced. These reactions and a positive nitroprusside reaction could be attributed to the products, particularly sulfide, formed as a result of the lability of gliotoxin in alkaline media.

Oxidizing agents such as permanganate, bromine water and sodium hypochlorite rapidly converted the sulfur of gliotoxin to sulfate. Reducing agents, including sodium sulfite, stannous chloride, hydriodic acid, aluminum amalgam, zinc or tin and acid, reduced the sulfur in gliotoxin to hydrogen sulfide. Certain of these procedures will be considered in detail in later papers. By treatment of gliotoxin with mercuric acetate or silver nitrate, *one* sulfur atom was removed. Cupric sulfate, lead acetate, and barium chloride gave no reaction.

A Zerewitinoff determination of active hydrogen in gliotoxin dissolved in pyridine gave somewhat uncertain results, for a rather large blank from the pyridine was observed, and while the blank was constant under given conditions, it was variable with time. It was necessary to use pyridine because gliotoxin is insoluble in the isoamyl ether

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(4) Weindling, *Phytopathology*, **26**, 1068 (1936).

(5) Herzig and Meyer, *Monatsh.*, **18**, 379 (1897).

(6) Clowes, *Ber.*, **32**, 2841 (1903).

used for preparing the Grignard reagent. Our conclusion from this determination is that gliotoxin has two, or perhaps three, active hydrogens.

In attempts to replace these active hydrogens by acyl groups, a solution of gliotoxin in pyridine was boiled with acetic anhydride and with benzoyl chloride, but only gums resulted. By conducting the reaction at room temperature, however, and adding water cautiously, a crystalline dibenzoate was secured. A di-*p*-bromobenzoate and a di-*p*-nitrobenzoate were similarly prepared in crystalline form. No crystalline acetyl derivative was obtained; but by a quantitative acetylation following the method of Shriner,[7] the introduction of one acetyl radical into gliotoxin was established. No reaction with *p*-toluenesulfonyl chloride could be discovered; neither did phthalic anhydride in pyridine or on fusion react with gliotoxin.

From this examination of the chemistry of gliotoxin, the presence of an indole structure appears probable in the original substance, although the yield of indole derivative was low. The ultraviolet absorption spectrum[1] supports this conception, and the consistent isolation of indole-2-carboxylic acid under different conditions of reaction makes it difficult to believe that this product is the result of a secondary reaction. A further study of the alkaline hydrolysis to determine whether the yield of indole derivative can be improved is desirable in order to help remove any doubt on this point. It is worth noting that the benzene ring in the indole nucleus is unsubstituted. This greatly reduces the number of possible locations of the other atoms in gliotoxin. Of the thirteen carbon atoms in the gliotoxin molecule, the indole-2-carboxylic acid fragment accounts for nine. One more is known to be present as a methyl radical attached to nitrogen. The disposition of the remaining three carbons, of two, possibly three, active hydrogens is still unknown; and the relation of the one other nitrogen and of the two sulfur atoms to the portion of the molecule thus far outlined also remains to be elucidated. In the following paper of this series, the position of these three carbon atoms is elucidated by means of a study of the reduction of gliotoxin by hydriodic acid. The arrangement of the functional groups and the nature of the sulfur atoms in gliotoxin will be the subject of subsequent papers.

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Experimental Part

Alkaline Degradation of Gliotoxin.—To a 10% solution of sodium hydroxide in a Kjeldahl distillation apparatus equipped with a separatory funnel and spray trap was added a weighed sample of gliotoxin. The solution was boiled for an hour and the volatile base collected in standard 0.1 *N* hydrochloric acid. The unused acid was determined by standard 0.1 *N* sodium hydroxide using methyl red as indicator. A receiver containing ammoniacal zinc sulfate was then substituted for that containing the standard acid. After addition of excess 15% sulfuric acid through the funnel, the liberated hydrogen sulfide was distilled into the zinc sulfate solution, the precipitated sulfide was treated with hydrochloric acid and determined iodometrically. The results are presented in Table I.

TABLE I

ALKALINE HYDROLYSIS OF GLIOTOXIN

Sample, mg.	% N volatile with base	% N by analysis[1]	% S volatile with acid	% S by analysis[1]
236.9	3.02	8.27	9.0	19.38
236.1	3.31	8.27	7.7	19.38
146.5	..	8.27	11.6	19.38
210.3	3.25[a]	8.27	..	19.38

[a] Hydrolysis by barium hydroxide.

A small amount of free sulfur steam distilled during the distillation of hydrogen sulfide and was identified by mixed melting point, 118°, with an authentic sample. From 450 mg. of gliotoxin, 130 mg. of a brick red residue insoluble in water or mineral acid remained. It was soluble in sodium hydroxide, contained nitrogen and sulfur, and did not melt, but merely charred gradually.

Concentration of a solution of the volatile base in dilute hydrochloric acid gave a white deliquescent solid melting at 222–225°. This was compared with an authentic sample of methylamine hydrochloride melting at 223–227°; no depression was observed on determination of the mixed melting point. Conversion to the chloroplatinate gave a yellow solid which was analyzed.

Anal. Calcd. for $C_2H_{12}Cl_6N_2Pt$: C, 6.4; H, 3.2; Pt, 41.3. Found: C, 6.12, 6.67; H, 2.96, 3.11; Pt, 41.2, 41.3.

By boiling 500 mg. of gliotoxin with 70 ml. of a 15% solution of barium hydroxide for one and one-half hours, followed by removal of the barium as sulfate, a clear yellow faintly acid solution was obtained. On evaporation to dryness, this yielded 80 mg. of a red-brown but crystalline residue. By subliming this material at 110–120° at 1 mm., 25 mg. of a white crystalline sublimate was secured. This substance melted at 201–202°, reacted with potassium permanganate and bromine water and contained nitrogen but not sulfur.

Anal. Calcd. for $C_9H_7NO_2$: C, 66.1; H, 4.35, N, 8.70. Found: C, 66.5; H, 4.26; N, 8.6.

By adding 5% silver nitrate to a solution of 5 mg. of the substance in water, a flocculent precipitate was obtained.

Anal. Calcd. for $C_9H_6AgNO_2$: Ag, 41.1. Found: Ag, 40.6.

These data give $C_9H_7NO_2$ as the best molecular formula.

The methyl and ethyl esters, each made from 5 mg. of sublimate by the action of the alcohol saturated with hydrogen chloride, melted at 149.5–151.5° and 121–123°, respectively. These data agree with those reported for indole-2-carboxylic acid and its esters,[8] and each of the three substances melted without depression on admixture with the corresponding authentic samples.

Oxidation by Bromine Water.—Into a 1% solution of gliotoxin in acetic acid bromine vapor was aspirated until a definite yellow orange color persisted. After two hours, a slight excess of barium chloride solution was added and the precipitate was digested at 50°, filtered, washed, ignited and weighed. The data are given in Table II.

Reaction of Gliotoxin with Mercuric Acetate and with Silver Nitrate.—To a solution of 50 mg. of gliotoxin in 25 ml. of absolute alcohol was added a solution of 200 mg. of

(7) Shriner, "Quantitative Analysis of Organic Compounds," Edwards Brothers, Inc., Ann Arbor, Michigan, 1938.

(8) Reissert, *Ber.*, **30**, 1045 (1897); Ciamician and Zatti, *ibid.*, **21**, 1931 (1888); Gränacher, Mahal and Gerö, *Helv. Chim. Acta*, **7**, 579 (1924). The ethyl ester reported by Oddo and Sessa, *Gass. chim. ital.*, **41**, 247 (1911), melting at 107° is not in agreement with an authentic sample prepared from indole-2-carboxylic acid synthesized by the method of Reissert. Our authentic ethyl ester melts at 121–122° and the methyl ester at 150–151°. These values agree with those reported by Gränacher.

TABLE II

OXIDATION BY GLIOTOXIN BY BROMINE WATER

Sample, mg.	$BaSO_4$, mg.	% S	% S (Carius)
97.8	138.5	19.46	19.37
133.7	190.9	19.60	19.37

mercuric acetate in 10 ml. of alcohol to which 10 drops of acetic acid was added to decrease hydrolysis. After standing in the dark for twenty-four hours, the amorphous, slowly-deposited white complex containing mercuric acetate and mercuric sulfide, when filtered, washed and dried, weighed 90 mg. corresponding to 5.22 mg. of sulfur or 53% of the total sulfur. The filtrate was treated with hydrogen sulfide to remove excess mercury, filtered and evaporated. A resinous sulfur-containing solid remained.

A solution of 100 mg. of gliotoxin in 25 ml. of absolute alcohol was treated with a solution of 350 mg. of silver nitrate in 25 ml. of 95% alcohol. A black precipitate of silver sulfide soon settled out, and a slight silver mirror formed. After an hour, the precipitate was collected on a filter. It weighed 80 mg., corresponding to 10.3 mg. of sulfur, or 53% of the total sulfur in the sample.

Determination of Active Hydrogen.—The sample, dissolved in 10 ml. of anhydrous pyridine, after standing an hour to ensure complete solution and temperature equilibrium, was treated with ethylmagnesium bromide in an apparatus similar to that of Soltys.[9] Gas was at once evolved. After fifteen to twenty minutes the gas evolution subsided and the reaction vessel was warmed at 50° with shaking for five minutes. After the original temperature was established, the volume increase was determined. The data in Table III show that the pyridine gave a high blank. The pyridine was dried over solid potassium hydroxide, kept over barium oxide for a week, and redistilled.[10] The constancy of the blank, determined on the same sample, permitted a reasonably satisfactory estimation of active hydrogen.

TABLE III

DETERMINATION OF ACTIVE HYDROGEN IN GLIOTOXIN BY REACTION WITH ETHYLMAGNESIUM BROMIDE

Sample, mg.	Volume increase, ml.	Active hydrogens
86.6	30.2 (28°, 733 mm. cor.)	2.2
109.7	38.0 (29°, 730 mm. cor.)	3.2
105.5	32.7 (29°, 730 mm. cor.)	2.2
0	15.4 (31°, 730 mm. cor.)	Blank
0	15.3 (29°, 730 mm. cor.)	Blank

The quantitative acetylation method of Shriner[7] was used to determine how many acetyl groups can be introduced into gliotoxin. Two samples of 100 mg. each were dissolved in 1.0 ml. of dry pyridine and 5 ml. of the acetyl chloride–toluene reagent was added. After twelve hours at room temperature water was added and the mixture titrated with 0.1 *N* sodium hydroxide. The two samples showed acetylation equivalent to 3.3 and 3.1 ml. of 0.1 *N* sodium hydroxide, respectively. The average equivalent weight from these data is 312. If the molecular weight is taken to be 326,[1] this represents 1.05 acetyl groups introduced per molecule. No crystalline acetate could be secured from these reactions.

A sample of 55 mg. of gliotoxin was treated with 2.0 ml. of 20% acetic anhydride in pyridine. Water was added after half an hour and the residual acetic acid determined by titration. The combined acetic acid, obtained by difference, was equivalent to 1.5 ml. of 0.1 *N* sodium hydroxide. The equivalent weight per acetyl group is then 366, representing 0.9 acetyl groups on the basis of a molecular weight of 326.

Crystalline Acylation Products of Gliotoxin.—A solution of 1 g. (3.07 millimoles) of gliotoxin in 10 ml. of dry pyridine was cooled in an ice-bath. During an hour 1.4 g. (10 millimoles) of benzoyl chloride was added with stirring. After standing for twelve hours at room temperature, the solution was red-brown and contained a deposit of pyridine hydrochloride. After cooling in an ice-bath, water was added dropwise with stirring. Crystallization was initiated by scratching. After one volume of water was added, the precipitate which formed was allowed to stand for an hour, filtered, washed with alcohol and dried to give 1.5 g. of product. Digestion with hot alcohol removed a yellow impurity. The product was recrystallized from hot chloroform by the addition of methanol, yielding nearly colorless glistening plates melting at 192–193° with decomposition; $[\alpha]^{23}D$ −20° (1% in $CHCl_3$).

Anal. Calcd. for $C_{27}H_{22}N_2O_6S_2$ (dibenzoate): C, 60.70; H, 4.12; N, 12.00; S, 5.25. Found: C, 60.02; H, 3.95; N, 12.26; S, 5.22.

The substance is sparingly soluble in acetone and alcohol, but soluble in pyridine and dioxane. In the latter solvent it appeared to undergo alteration since the specific rotation varied. Recrystallization from acetic acid also caused alteration since the melting point was lowered to 186–188°. The dibenzoate gave no reaction with acetyl chloride in pyridine.

The di-*p*-bromobenzoate of gliotoxin was prepared by a similar procedure from 1 g. of gliotoxin in pyridine and 1.5 g. of *p*-bromobenzoyl chloride. The product crystallized from dioxane solution on addition of water to give 1.8 g. of colorless needles melting at 193° with decomposition; $[\alpha]D$ +20° (1% in $CHCl_3$).[11]

Anal. Calcd. for $C_{27}H_{20}Br_2N_2O_6S_2$ (di-*p*-brombenzoate): C, 46.80; H, 2.90; Br, 23.10. Found: C, 46.50; H, 2.72; Br, 24.17.

The di-*p*-nitrobenzoate was prepared from 326 mg. (1 millimole) of gliotoxin dissolved in pyridine and 930 mg. (5 millimoles) of *p*-nitrobenzoyl chloride in the manner previously described. The product was insoluble in alcohol, ether, or acetone. It crystallized from chloroform, on addition of methanol, in pale yellow prismatic rods which melted with decomposition at 189°; $[\alpha]^{23}D$ +13° (1% in $CHCl_3$).

Anal. Calcd. for $C_{27}H_{20}N_4O_{10}S_2$ (di-*p*-nitrobenzoate): N, 9.0; S, 10.25. Found: N, 9.2; S, 9.84. Calcd. for $C_{34}H_{23}N_5O_{13}S_2$ (tri-*p*-nitrobenzoate): N, 9.05; S, 8.28.

Summary

An investigation of the chemical behavior of gliotoxin has shown that a part of the carbon skeleton probably includes that of indole-2-carboxylic acid. The presence of an N-methyl grouping has been demonstrated. The lability of the sulfur atoms in gliotoxin makes it difficult to secure crystalline reaction products. A number of reactions by which sulfur can be removed have been discovered, including oxidation by bromine water, reaction with mercuric acetate and silver nitrate, and reduction by various acidic and neutral reducing agents. Three crystalline derivatives of gliotoxin have been prepared: the dibenzoate, the di-*p*-bromobenzoate and the di-*p*-nitrobenzoate. In each case the formulation of the product agrees with the formulation of gliotoxin previously proposed.

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(9) Soltys, *Mikrochemie*, **20**, 107 (1936).

(10) MacArdle, "Solvents in Organic Chemistry," D. Van Nostrand Co., New York, N. Y., 1925, p. 108.

(11) In a preliminary experiment, a smaller sample of a product was obtained for which the melting point, 189–191°, and specific rotation, $[\alpha]D$ +20° (1% in dioxane), agree with those given above. The analytical values, however, indicated that the substance was a tri-*p*-bromobenzoate. *Anal.* Calcd. for $C_{34}H_{23}Br_3N_2O_7S_2$ (tri-*p*-bromobenzoate): C, 46.60; H, 2.63; Br, 27.40; N, 3.20; S, 7.32. Found: C, 46.00; H, 2.62; Br, 27.17; N, 3.07, 3.65; S, 7.91. All subsequent preparations, regardless of the amount of *p*-bromobenzoyl chloride used, yielded only the di-*p*-bromobenzoate.